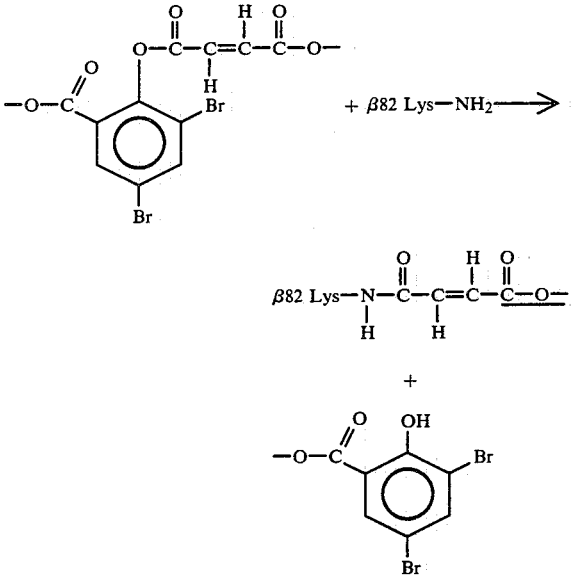
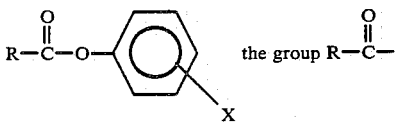


The reaction conditions are the same as described in Example 1, except that hemoglobin is maintained in the oxy form. Normal ambient partial pressures of oxygen in room air are sufficient for this purpose. With a 1.5 molar excess of the reagent over hemoglobin the yield of the product was approximately 20%. The derivative was purified by chromatography on DEAE cellulose as described in the previous example. Two-dimensional gel electrophoresis of the modified hemoglobin showed that both of the beta chains were modified. X-ray crystallographic studies showed that the site of modification was at Lys 82 of the beta chains. This was confirmed by tryptic peptide mapping. The oxygen affinity of the modified hemoglobin is decreased by approximately 1.6 fold. At a pH of 7.0 in 50 mM Bis-Tris buffer, the P_{50} was found to be increased from 7.9 mmHg for native hemoglobin to 12.9 mmHg for the modified derivative.

The reaction of mono(3,5-dibromosalicyl) fumarate with the alpha-alpha cross-linked derivative described in Example 1 under oxygenated conditions, occurs similarly to native hemoglobin. Correspondingly, oxygen binding studies as those in FIG. 2 show that the oxygen affinity of the alpha-alpha cross-linked derivative is further decreased by the addition of the negatively charged carboxylate group within the DPG binding site. In principle, a number of different derivatives could be prepared, having a range of oxygen affinities, by modification of the alpha-alpha cross-linked derivative with analogs of mono(3,5-dibromosalicyl) fumarate. The resulting oxygen affinity will depend on the negatively charged group which is added within the DPG binding site. In the following general structure:



becomes covalently attached to the protein. The number and type of negatively charged substituents within this group may be varied. In addition to the carboxyl group these would include phosphonate, phosphate, sulfonate, and sulfate groups. In general, the greater the number of negative charges on the attached group, the lower would be the oxygen affinity of the modified hemoglobin.

In summary, the results of the studies performed demonstrate that the alpha-alpha intramolecularly cross-linked hemoglobin, described herein has the properties of an effective blood substitute, plasma expander and in general can be used for this purpose where conventional donor blood samples are now used.

Other modifications may be made without necessarily departing from the scope and spirit of the invention, the important factor and contribution being the discovery and use and technique for the alpha-alpha cross-linked modified hemoglobin.

It can further be seen that the invention accomplishes at least all of the objectives heretofore stated.

What is claimed is:

1. A pharmaceutical composition for use as a blood substitute and blood plasma expander comprising a therapeutically effective amount of Lys 99 $Alpha_1$ to Lys 99 $Alpha_2$ intramolecularly cross-linked, stroma-free hemoglobin, substantially free of hemoglobin modified at other sites, soluble in aqueous and physiological fluids and capable of reversibly binding oxygen, and a pharmaceutically acceptable carrier.

2. The composition of claim **1** wherein said alpha-alpha cross-link is with an amino group-specific cross-linking agent.

3. The composition of claim **2** wherein said cross-linking agent is an acylating agent.

4. The composition of claim **3** wherein said cross-linking agent is a diester cross-linking agent.

5. The composition of claim **6** wherein said cross-linking agent is a phenyl ester cross-linking agent.

6. The composition of claim **7** wherein said cross-linking agent is bis(3,5-dibromosalicyl) fumarate.

7. The composition of claim **1** wherein the carrier is liquid and the composition contains from about 1% to about 10% of said hemoglobin.

8. The composition of claim **1** wherein said hemoglobin is also modified with a second reagent introducing a negatively charged group at the 2,3-diphosphoglycerate binding site.

9. The composition of claim **8** wherein said reagent is mono(3,5-dibromosalicyl) fumarate.

10. A method of replacing or increasing the circulating blood volume or increasing oxygen delivery to tissues in man or animal species comprising:

transfusing into the blood circulatory system a blood volume expander which consists essentially of Lys 99 $Alpha_1$ to Lys 99 $Alpha_2$ intramolecularly cross-linked, stroma-free hemoglobin which is substantially free of hemoglobin derivatives modified at other sites, having a molecular weight of about 64,000 in an isotonic solution.

11. The method of claim **10** wherein the hemoglobin is also modified with a negatively charged group at the 2,3-diphosphoglycerate binding site.

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